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A CCR4 antagonist ameliorates atopic dermatitis-like skin lesions induced by dibutyl phthalate and a hydrogel patch containing ovalbumin

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ABSTRACT

CCR4 is a chemokine receptor highly expressed by Th2 cells, and regarded as a potential therapeutic target for atopic dermatitis (AD). CCL17 and CCL22 are the CCR4 ligands, and thymic stromal lymphopoietin (TSLP) is shown to promote the expression of CCL17 and CCL22 by dendritic cells. Here, by using dibutyl phthalate (DBP), a TSLP inducer, and a hydrogel patch as a transcutaneous delivery device for ovalbumin, we developed a novel murine AD model and investigated the effect of Compound 22, a CCR4 antagonist. We first found that the mRNA expression of TSLP together with CCL17 and CCL22 was increased in the skins treated with DBP. Furthermore, the topical application of ovalbumin and DBP efficiently and rapidly induced AD-like skin lesions in BALB/c mice, which were characterized by ear swelling accompanied by infiltration of eosinophils, mast cells, and CCR4-expressing Th2 cells in the skin lesions, and elevated total IgE levels in the sera. Using this AD model, we demonstrated that cutaneous administration of Compound 22 inhibited Th2 cell infiltration and ameliorated the AD-like skin lesions. These results suggest that our AD model could be useful for studying new therapeutic strategies. Collectively, CCR4 antagonists may be a promising approach for treating AD.

1. Introduction

Atopic dermatitis (AD) is a chronic inflammatory skin disease characterized by pruritus and relapsing eczema, and is frequently associated with high serum IgE levels and eosinophilia [1,2]. In addition, most AD patients have an increased number of T-helper (Th) 2 cells in the skin lesions and peripheral blood [1–4]. Th2 cells produce IL-4, IL-5, and IL-13, which are responsible for the promotion of IgE production by B cells. Furthermore, mast cell-bound IgE leads to mast cell degranulation and initiates allergic reactions. CCR4 is a major trafficking receptor for Th2 cells, and its ligands are TARC/CCL17 and MDC/CCL22 [5]. Indeed, CCR4 is shown to be expressed on infiltrated Th2 cells in skin lesions of AD patients [6]. Furthermore, we and others have shown that the serum levels of CCL17 and CCL22 are highly elevated in

AD patients and correlate positively with disease severity [7–9]. In this context, a CCL17 ELISA kit (Alaport TARC) has been developed to objectively monitor the disease activity and therapeutic response of AD patients. Thus, the CCR4 axis is now considered to play a pivotal role in the pathogenesis of AD by recruiting CCR4-expressing Th2 cells.

Thymic stromal lymphopoietin (TSLP) is highly expressed by keratinocytes in skin lesions of acute and chronic AD [10,11], and induces the expression of Th2-type cytokines, such as IL-4, IL-5, and IL-13, and Th2-attracting chemokines, such as CCL17 and CCL22, in dendritic cells (DCs) [11,12]. Furthermore, it was reported that TSLP acted directly on naïve CD4 + T cells to promote Th2 differentiation during allergic inflammation in the skin [13]. Thus, TSLP is thought to be involved in the induction of Th2 responses and the development of Th2-type allergic diseases such as AD.

Abbreviations: AD, atopic dermatitis; DBP, dibutyl phthalate; DC, dendritic cell; OVA, ovalbumin; Th, T-helper; Treg, regulatory T; TSLP, thymic stromal lymphopoietin

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Recently, dibutyl phthalate (DBP) was reported to induce TSLP expression in keratinocytes of the skin and to be an adjuvant for FITC-induced contact hyper sensitivity [14–16]. In addition, we developed a hydrogel patch as an efficient transcutaneous immunization system, and demonstrated that the hydrogel patch can efficiently promote skin penetration of antigenic proteins [17–19]. Furthermore, by using the hydrogel patch containing ovalbumin (OVA), we were able to induce acute AD-like skin lesions only by three weeks in BALB/c mice [20]. In the present study, we therefore combined DBP and the hydrogel patch to develop a more efficient model of AD. Using this AD model, we also demonstrated that cutaneous administration of Compound 22, a CCR4 antagonist, ameliorated the AD-like skin lesions.

2. Materials and methods

2.1. Mice

BALB/c mice were purchased from Japan SLC (Shizuoka, Japan). Mice were maintained in specific pathogen-free conditions. All animal experiments were approved by the Center of Animal Experiments, Kindai University, and performed in accordance with the institutional guidelines.

2.2. Hydrogel patch formulation

The hydrogel patch formulation, comprising cross-linked HiPAS™ acrylate medical adhesives (CosMED Pharmaceutical Co. Ltd., Kyoto, Japan): octyldodecyl lactate:glycerin:sodium HA = 100:45:30: 0.2 as weight ratio of composition, was prepared as described previously [17–19].

2.3. The AD model

BALB/c mice were sensitized on days 0 and 7 by an intraperitoneal injection of 25 µg OVA and 1 mg of alum (Imject Alum; Thermo Fisher Scientific, Waltham, MA) in 300 µl of PBS. On days 14, 19, and 24, a hydrogel patch (1 × 2 cm²) containing DW or OVA (100 µg) was applied to the auricle skin, covered with a wound management film to allow for better skin adherence, and left for 24 h. The mouse skin was treated with DBP solution in Tween 80 (1:1 ratio) every day during the AD induction period. The detailed protocols are shown in Fig. 1A. Skin and blood samples were collected on day 26 (Fig. 1A). Compound 22, a small molecule CCR4-specific antagonist, was synthesized based on the published information as described previously [21].

2.4. Histochemistry

Skin sections were stained with HE as described previously [22]. Acanthosis was evaluated by measuring the epidermal thickness on the ear skin sections. The skin sections were also stained with toluidine blue as described previously [20]. We counted mast cells between the cartilage and squamous epithelium in five random sites (×400) of a toluidine blue-stained ear section under a light microscope to obtain the mean cell number per site.

2.5. ELISA

Serum total IgE levels were measured using an ELISA kit purchased from Biolegend (San Diego, CA) and following the manufacturer's instructions.

2.6. Isolation of cells

Skin samples were incubated for 60 min at 37 °C in RPMI1640 supplemented with 0.24 mg/ml collagenase A (Roche; Basel, Switzerland), and 40 U/ml DNase I (Thermo Fisher Scientific). After

shaking vigorously, the resulting suspensions were filtered through a 70-µm cell strainer. Splenocytes were obtained by mashing spleens through a 70-µm cell strainer, followed by erythrocyte lysis with an ACK lysis buffer (150 mM NH₄Cl, 10 mM KHCO₃ and 0.1 mM Na₂EDTA, pH 7.2).

2.7. Flow cytometric analysis

We purchased fluorescence-labeled anti-CCR4 (clone 2G12), anti-CCR3 (clone J073E5), anti-IL-4 (clone 11B11), anti-CD45 (clone 30-F11), and anti-CD4 (clone GK1.5) from BioLegend and anti-siglec-F from BD Biosciences (San Diego, CA). Cells were incubated for 30 min with a mixture of anti-CD45 and anti-CD4, anti-CCR4, anti-CCR3, or anti-siglec-F. For intracellular staining, cells were then fixed and permeabilized (Cytofix/Cytoperm kit; BD Biosciences) and subsequently stained intracellularly with anti-IL-4. After washing, cells were immediately analyzed on a BD LSR Fortessa (BD Biosciences) and analyzed with the FlowJo software (Tree Star Inc., Ashland, OR). For IL-4 staining, cells were stimulated with phorbol 12-myristate 13-acetate and ionomycin for 4 h.

2.8. Real-time PCR

Quantitative real-time PCR was performed on an ABI7000 (Applied Biosystems, Foster City, CA) using a Kapa SYBR Fast qPCR Kit (Kapa Biosystems, Woburn, MA) according to the manufacturer's protocol. The PCR conditions were 60 °C for 20 min, 94 °C for 5 min, and then 40 cycles of 94 °C for 30 s (denaturation) and 55 °C for 30 s (annealing extension). The primers for IL-4, IL-17A, IFN-γ, CCL17, CCL22, and GAPDH were described previously [20]. The other primers used were as follows: +5'-CAGCTAGTTGTCATCCTGGTCTTC-3' and —5'-GCCGATGATGCTCTCAAGTGA-3' for TSLP.

2.9. Statistical analysis

The procedure has been described previously [23]. Briefly, Student's *t*-test was performed to analyze differences between the two groups. One-way analysis of variance with the Holm post hoc test was performed for multiple groups. We considered *P* < 0.05 as statistically significant.

3. Results

3.1. DBP induces TSLP, CCL17, and CCL22 expression in skin lesions of BALB/c mice

Our previous studies have shown that a hydro gel patch promotes skin penetration of antigenic proteins and induces antigen-specific Th2 responses more efficiently than a gauze patch [17–19]. Therefore, we used the hydrogel patch as a transcutaneous delivery device for OVA as an antigen. BALB/c mice were sensitized with OVA and alum, which elicits antigen-specific IgE production, and then the hydrogel patch containing OVA with or without DBP treatment was applied to the auricle skin (Fig. 1A). Because DBP was reported to induce the expression of TSLP, which is an inducer of Th2-attracting chemokines CCL17 and CCL22 [11,12,14–16], we first examined the effect of DBP on the expression of TSLP, CCL17, and CCL22 in the skin lesions. As shown in Fig. 1B, DBP treatment alone significantly increased the expression levels of not only TSLP mRNA but also CCL17 and CCL22 mRNAs in the skin lesions. Furthermore, the combination of the hydrogel patch containing OVA and DBP treatment further increased the expression levels of TSLP, CCL17, and CCL22 mRNAs in the skin lesions.

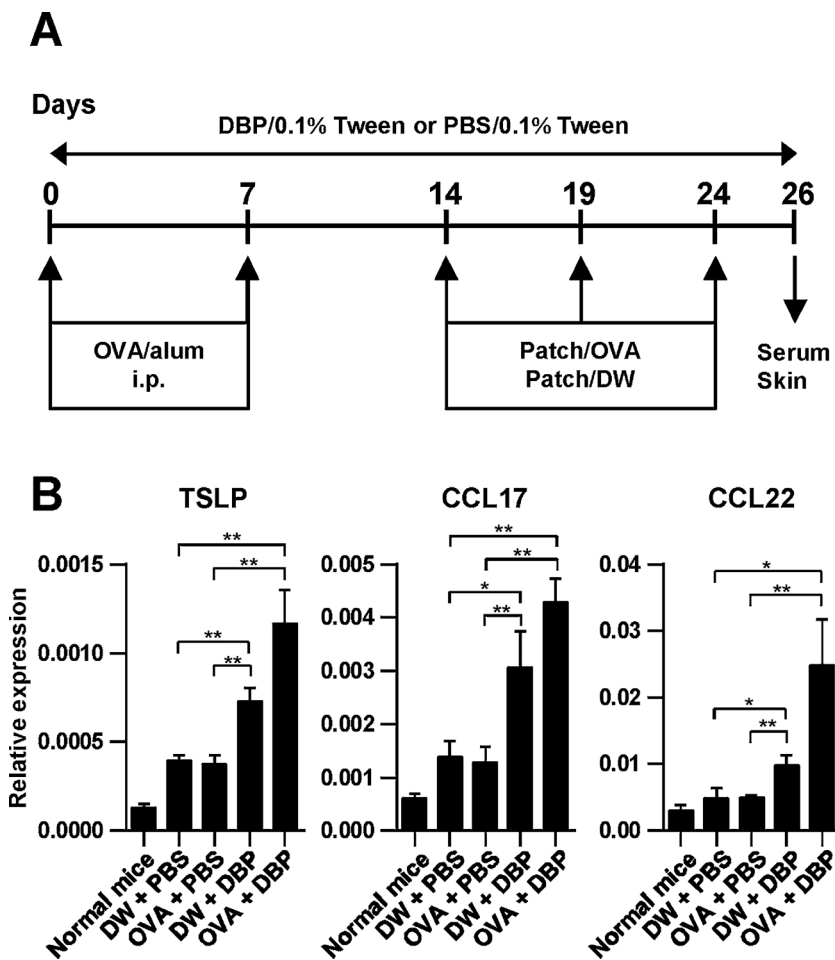


Fig. 1. Induction of TSLP, CCL17, and CCL22 expression by a hydrogel patch containing OVA and DBP treatment. (A) The experimental protocol for induction of allergic skin inflammation. BALB/c mice were sensitized on days 0 and 7 with an intraperitoneal injection of OVA and alum. On days 14, 19, and 24, a hydrogel patch containing DW or OVA was applied to the auricle skin. The auricle skin was treated with DBP every day during the AD induction period. Skin and serum samples were collected on day 26. (B) The mRNA expression of TSLP, CCL17, and CCL22 in skin lesions was examined by real-time PCR. Data are expressed as the mean \pm SE of results from nine mice. * P < 0.05 and ** P < 0.01.

3.2. Topical application of OVA and DBP efficiently induces allergic skin inflammation in BALB/c mice

Next, we histologically evaluated the skin lesions induced by the hydrogel patch containing OVA and DBP treatment. HE staining demonstrated increased ear swelling and epidermal hyperplasia in the skin applied with the hydrogel patch containing OVA and DBP treatment (Fig. 2A and B). In this context, we observed increased infiltration of inflammatory cells in the skin lesions induced by the hydrogel patch containing OVA and DBP treatment compared with PBS, OVA, or DBP treatment alone (Fig. 2A). Using flow cytometry, we also analyzed cells infiltrating the skin lesions. Th2 cells (IL-4+CD4+ cells) and eosinophils (CCR3+siglec-F+ cells) were highly increased in the skin lesions induced by the hydrogel patch containing OVA and DBP treatment (Fig. 2C). In addition, we confirmed that CCR4 was indeed expressed on Th2 cells infiltrating in the skin lesions (Fig. 2D). Furthermore, toluidine blue staining demonstrated significantly increased infiltration of mast cells in the skin lesions (Fig. 2C). We also measured total IgE in the blood samples of these mice. As shown in Fig. 2E, the combination of the hydrogel patch containing OVA and DBP treatment markedly increased total IgE levels.

3.3. Topical application of OVA and DBP induces acute AD-like skin lesions in BALB/c mice

AD is a biphasic disease, including the acute phase predominated by Th2 cytokines and the chronic phase predominated by Th1 cytokines [24]. It has further been reported that IL-17A is expressed only in acute AD skin lesions [25], and IL-22 is expressed in both acute and chronic AD skin lesions [26]. We therefore examined the expression of IL-4, IL-

17A, IL-22, and IFN- γ in the skin lesions using real-time PCR. IL-4 and IL-17A mRNAs, but not IFN- γ mRNA, were significantly increased in the skin lesions induced by the hydrogel patch containing OVA and DBP treatment (Fig. 3). In addition, IL-22 mRNA was significantly increased in the skin lesions induced by the hydrogel patch containing OVA and DBP treatment. Because CCR4 is also expressed by regulatory T (Treg) cells [27], we examined the expression of Foxp3 mRNA, the Treg cell marker. Foxp3 mRNA was not significantly increased in any skin lesions, including those induced by the hydrogel patch containing OVA and DBP treatment (data not shown). Collectively, these results demonstrate that the combination of the hydrogel patch containing OVA and DBP treatment is able to efficiently induce allergic skin inflammation with features of skin lesions similar to the acute phase of human AD.

3.4. Compound 22, a CCR4 antagonist, ameliorates AD-like skin lesions

To examine the role of CCR4 in the present AD model, we used Compound 22, a selective CCR4 antagonist. We previously demonstrated that Compound 22 showed no cross reactivity to almost all human and mouse chemokine receptors by using a panel of murine L1.2 cells that stably express human and mouse chemokine receptors [21]. As shown in Fig. 4A, the cutaneous administration of Compound 22 dose-dependently inhibited Th2 cell infiltration into the AD-like skin lesions induced by the topical application of OVA and DBP. Compound 22 at 100 and 500 μ g/site also significantly inhibited eosinophil infiltration into the AD-like skin lesions (Fig. 4A). Consequently, Compound 22 dose-dependently reduced the ear and epidermal thickness (Fig. 4B and C). We also measured total IgE in the blood samples of these mice. As shown in Fig. 4D, Compound 22 at 500 μ g/site

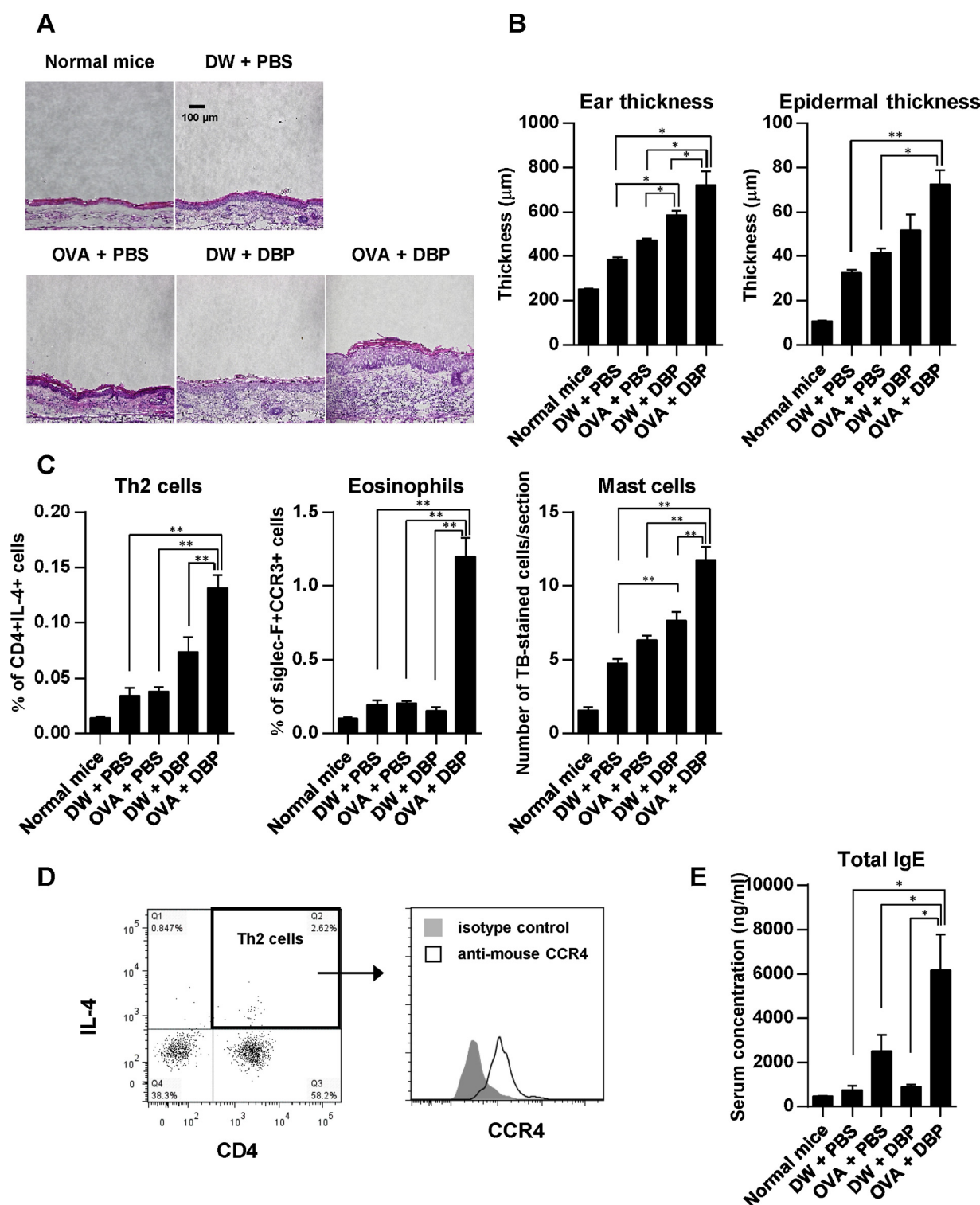


Fig. 2. Allergic skin inflammation induced by a hydrogel patch containing OVA and DBP treatment. (A) HE staining. (B) Ear and epidermal thickness in the skin. Data are expressed as the mean \pm SE of results from six mice. (C) Cells in the inflammatory skin were analyzed by flow cytometry using the CD45 gate. Th2 cells: CD4+IL-4+; Eosinophils: CCR3+siglec-F+. Mast cell numbers were determined by toluidine blue staining. Data are expressed as the mean \pm SE of results from six mice. (D) CD4⁺IL-4⁺ Th2 cells were analyzed for CCR4 expression by flow cytometry. The representative data are shown from at least three independent experiments. (E) Quantification of serum total IgE. Data are expressed as the mean \pm SE of results from seven mice. * P < 0.05 and ** P < 0.01. Scale bar = 50 μ m.

significantly reduced the elevated total IgE levels. These results demonstrate that CCR4 antagonists may prevent or treat AD-like skin lesions.

3.5. Effect of Compound 22 on AD-related gene expression in AD-like skin lesions

Th2-attracting chemokine CCL17 is regarded as a valuable biomarker for AD, and correlates with disease severity in AD [7,8].

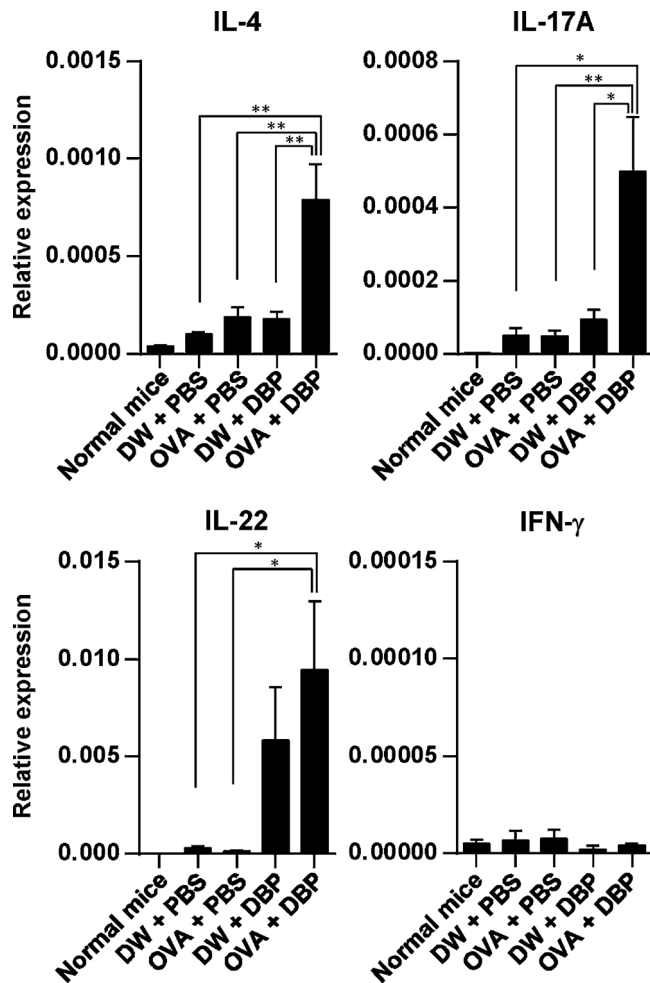


Fig. 3. Cytokine expression in AD-like skin lesions. The mRNA expression of IL-4, IL-17 A, IL-22, and IFN- γ in skin lesions was examined by real-time PCR. Data are expressed as the mean \pm SE of results from nine mice. * P < 0.05 and ** P < 0.01.

Therefore, we examined the effect of Compound 22 on the expression of AD-related genes, such as Th2-attracting chemokines and inflammatory cytokines, in the AD-like skin lesions induced by the topical application of OVA and DBP. As shown in Fig. 5, Compound 22 dose-dependently reduced the elevated expression of Th2-attracting chemokines, such as CCL17 and CCL22, in the AD-like skin lesions. Simultaneously, Compound 22 at 500 μ g/site significantly reduced the elevated expression of inflammatory cytokines, such as TSLP, IL-4, and IL17 A.

4. Discussion

In this study, we combined DBP treatment and a hydrogel patch as a transcutaneous delivery device for OVA in the classical OVA-sensitized mouse model. We found that the topical application of OVA and DBP efficiently induced AD-like skin lesions in BALB/c mice. Furthermore, we demonstrated the therapeutic effect of Compound 22, a CCR4 antagonist, on this AD mouse model.

Recently, it has been reported that DBP induces TSLP expression in the skin and acts as an adjuvant for Th2 responses [14–16]. The induction of TSLP expression is also considered to be one of the most important mechanisms leading to the adjuvant effect of DBP. Accordingly, TSLP has been shown to induce several allergy-related genes, including Th2-type cytokines and Th2-attracting chemokines, and is now regarded as a master regulator of Th2 inflammatory responses in allergic diseases. In this study, we have corroborated previous findings

and demonstrated that the addition of DBP treatment promotes the development of AD-like skin lesions by the hydrogel patch containing OVA; the expression of TSLP together with IL-4, CCL17, and CCL22 (Figs. 1B and 3); the increase in ear and epidermal thickness (Fig. 2A and B); the production of total IgE (Fig. 2D); and the infiltration of Th2 cells, eosinophils, and mast cells (Fig. 2C). The induction of TSLP expression seems to be highly important for the induction of CCL17 and CCL22 and the subsequent infiltration of CCR4-expressing Th2 cells in AD skin lesions. Thus, TSLP as an effective adjuvant may be potentially useful for developing animal models for allergic diseases.

The OVA-sensitized mouse model is one of the most standard models of AD. Repeated sensitization with a gauze patch containing OVA induces allergic skin inflammation, which has pathological features similar to the acute phase of human AD [28–30]. However, this model using the gauze patch requires multiple sensitizations over an extended period of time (usually 7 weeks) to induce the AD-like skin lesion. More recently, we have developed an AD mouse model using a hydrogel patch that can efficiently promote skin penetration of antigenic proteins and *Staphylococcus aureus* δ -toxin as a mast cell activator to shorten the period necessary to induce AD-like lesions [20]. Although the topical application of OVA and δ -toxin by the hydrogel patch induced AD-like skin lesions by three weeks in BALB/c mice, the AD-like skin lesions exhibited relatively mild inflammatory responses without significant hyperplasia. Therefore, in this study, we combined DBP as a TSLP inducer in our previous model using the hydrogel patch, and were able to induce more severe AD-like skin lesions with hyperplasia. IL-22 is a key regulator of keratinocyte differentiation and epidermal hyperplasia in skin diseases such as AD and psoriasis [31]. IL-22 is also preferentially produced by Th17 cells, although IL-22 is produced by a unique subset of cells, named Th22 cells, in humans but not in mice [31]. Indeed, in the present study, the expression levels of IL-22 and IL-17 were increased in AD-like skin lesions (Fig. 3), and the expression levels of IL-22 and IL-17 were much higher than those in our previous model using δ -toxin [20]. Thus, the infiltration of Th17 cells expressing IL-22 and IL-17 may be involved in aggravation of AD-like skin lesions including hyperplasia in the present AD model. Furthermore, it has been reported that Th17 cells, as well as Th2 cells, dominantly express CCR4 [5,32,33], and CCL17, but not CCL22, is expressed in endothelial cells of AD skin lesions [8,34]. Thus, CCL17 seems to be primarily responsible for the infiltration of Th17 cells into AD skin lesions. Consistent with this notion, although we found significantly increased expression of CCL17 in the AD-like skin lesions (Fig. 1), no marked difference was observed in the expression levels between the present AD model and our previous model using δ -toxin [20]. In this regard, because Th17 cells express CCR6 in addition to CCR4 [35], CCR6 may also be associated with the infiltration of Th17 cells. The role of CCR6 in the present AD model remains to be elucidated.

We have previously shown that serum CCL17 levels positively correlate with disease severity in AD patients [7,8]. CCL17 is now regarded as a useful biomarker to monitor AD disease activity during therapy. Furthermore, we have demonstrated that CCL17 is involved in the development of AD-like skin lesions using CCL17-transgenic mice and CCR4-deficient mice on the BALB/c background [20,36]. However, initial studies employing CCR4-deficient mice on the C57BL/6 background only demonstrated a minor role of CCR4 in the development of AD-like skin lesions [27,37]. One reason for the discrepancy between the previous studies and our studies may probably be due to the difference in the genetic background of CCR4-deficient mice. In addition, because these previous studies used the classical OVA-sensitized model using the gauze patch, we have also compared the expression levels of CCL17 in AD-like skin lesions between the present AD model and the classical model using the gauze patch. As a result, the expression levels of CCL17 in the classical model were much lower than those of our present model (data not shown). Thus, the discrepancy also may be partly due to the procedures used for the induction of AD-like skin

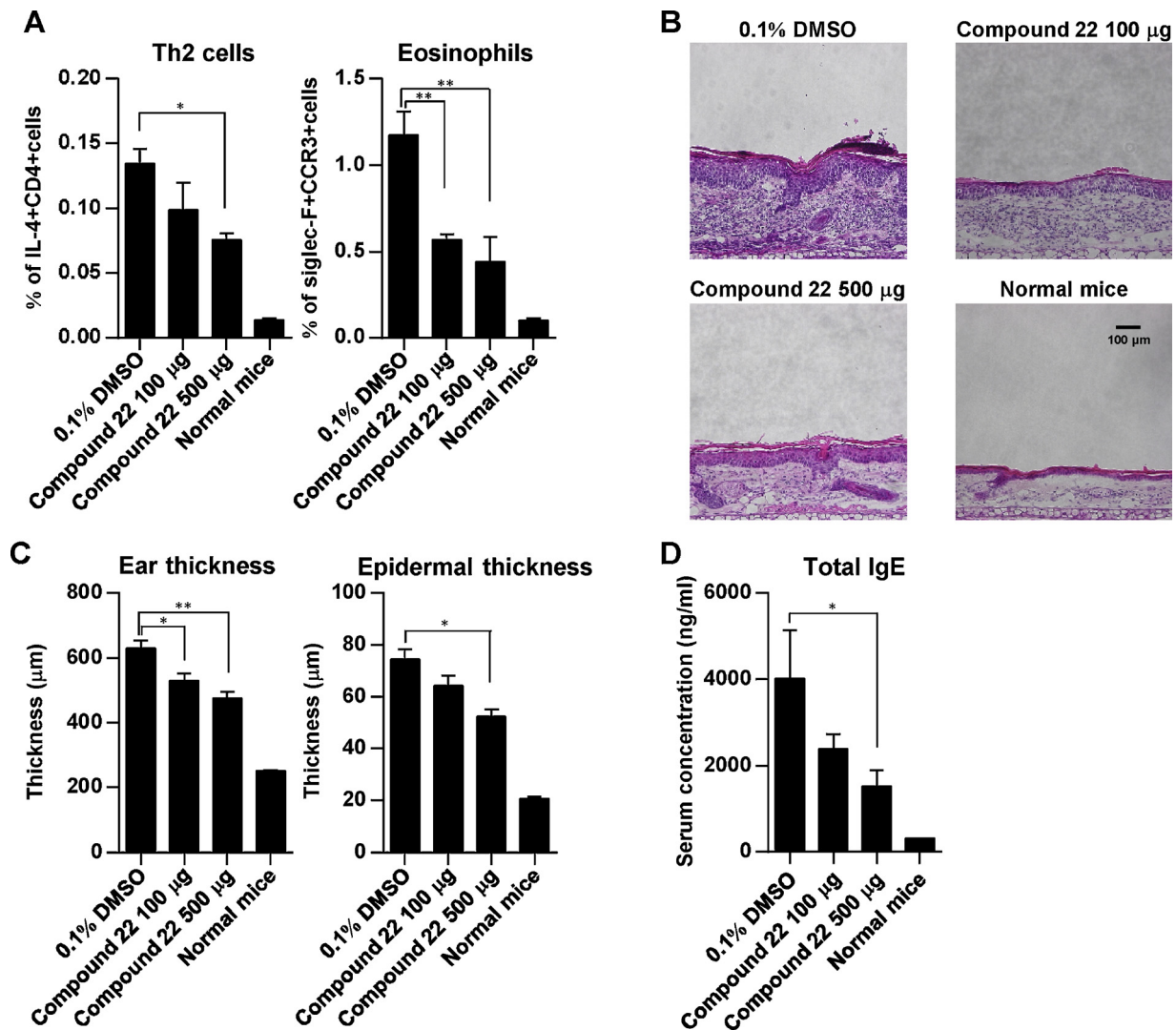


Fig. 4. Effect of Compound 22 on allergic skin inflammation. AD-like skin lesions were induced by a hydrogel patch containing OVA and DBP treatment. Compound 22 was transcutaneously applied to the skin every day. One day after the last application, skin and serum samples were obtained. (A) Cells in the inflammatory skin were analyzed by flow cytometry using the CD45 gate. Th2 cells: CD4+IL-4+; Eosinophils: CCR3+siglec-F+. Data are expressed as the mean \pm SE of results from four mice. (B) HE staining. (C) Ear and epidermal thickness in the skin. Data are expressed as the mean \pm SE of results from eight mice. (D) Quantification of serum total IgE. Data are expressed as the mean \pm SE of results from six mice. * P < 0.05 and ** P < 0.01. Scale bar = 50 μ m.

lesions.

AD is currently considered a biphasic disease with a predominant Th2 response in the acute phase and a switch to a Th1 response in the chronic phase [24]. In the acute phase, Th2 cells are predominantly found in the skin lesions and produce IL-4, IL-5, and IL-13, which are responsible for IgE production by B cells [24]. In the chronic phase, Th1 cells are increased in the skin lesions and produce IFN- γ , which contributes to dermal thickness and hyperkeratosis [24]. These proinflammatory cytokines are considered to play a prominent role in AD pathogenesis and to be potential therapeutic targets. Indeed, Dupilumab, an anti-IL-4/13R mAb, has recently been approved by the FDA for the treatment of moderate-to-severe AD, and tralokinumab, an anti-IL-13 mAb has also been tested in a phase study [38]. In the present study, we have demonstrated that a CCR4 antagonist ameliorates AD-like skin lesions by inhibiting the infiltration of Th2 cells which produce the Th2-type cytokines (Figs. 4 and 5). At any rate, because our present AD model represents an acute AD model, the CCR4 axis is likely to play a pivotal role at least in the acute phase of AD. Furthermore, Mogamulizumab, an anti-CCR4 mAb, has recently been reported to be tested in a phase study to treat patients with asthma [39]. Thus, CCR4 may be a potent therapeutic target for allergic diseases, including AD and

asthma.

In conclusion, we have demonstrated that the combination of the hydrogel patch containing OVA and DBP treatment efficiently induces allergic skin inflammation with skin lesions similar to those seen during the acute phase of human AD only by three weeks in BALB/c mice. Using this model, we have also demonstrated that cutaneous administration of the CCR4 antagonist ameliorates the AD-like skin lesions. Thus, our AD model employing the hydrogel patch may be potentially useful for both basic studies of AD and developing new therapeutic agents.

Conflict of interest

The authors indicated no potential conflicts of interest.

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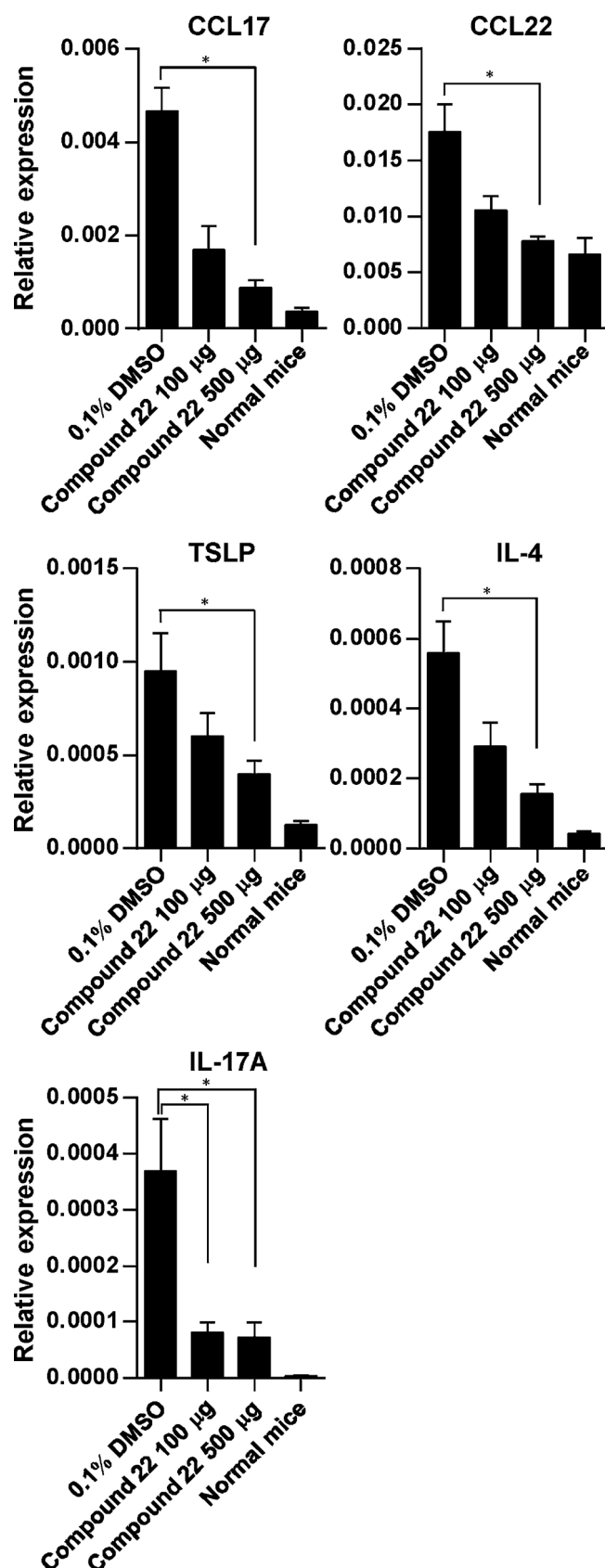


Fig. 5. Effect of Compound 22 on the expression of Th2-attracting chemokines and inflammatory cytokines. AD-like skin lesions were induced by a hydrogel patch containing OVA and DBP treatment. Compound 22 was transcutaneously applied to the skin every day. One day after the last application, skin samples were obtained. The mRNA expression of CCL17, CCL22, TSLP, IL-4, and IL-17 A in skin lesions was examined by real-time PCR. Data are expressed as the mean \pm SE of results from four mice. * $P < 0.05$.

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